

Characterization of Cytotoxic Effect of Asbestos on Normal Human Fibroblasts

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Using viable cell counting and the incorporation of radioactive thymidine in cell TCA precipitate, we have attempted to separate the effects of asbestos on cell growth inhibition (reproductive failure) vs. cell killing. NIEHS crocidolite and NIEHS intermediate-sized chrysotile at a concentration of 10 $\mu\text{g/mL}$ were studied. Cytotoxic effects were demonstrated with both types of asbestos. Thymidine incorporation expressed as cpm/viable cell was enhanced in the presence of either form of asbestos. It is concluded that both negatively charged crocidolite and positively charged chrysotile are toxic to human fibroblast cells in culture and exert their cell killing but not growth inhibition effect concurrently with a stimulation of DNA synthesis.

Introduction

Various *in vitro* studies have demonstrated the cytotoxic effect of asbestos exposure on different cell types (1-5). Asbestos-induced changes in short-term viable cell counts and/or total DNA content per culture have been interpreted to be the result of growth inhibition (1, 2) and asbestos-modified conventional colony-forming efficiency studies, which are expressed as percent survival, usually imply a reproductive delay or failure. Previous studies on DNA synthesis and cellular proliferation have either attempted to assay total radioactive thymidine incorporation per sample over a prolonged period of time (3) or measure DNA content per dish (2). In either case, the loss of DNA and/or incorporated radioactivity in the killed, detached, and washed away cells was not corrected for. In the present study, the trypan blue exclusion method was used to assess the cytotoxic effect of crocidolite and chrysotile exposure, asbestos fibers with different potentials to induce carcinoma in humans (6, 7), on normal human fibroblasts. The effect of the exposure on DNA synthesis was also investigated by

measuring radioactive thymidine incorporation per viable cell for the last hour of asbestos treatment.

Materials and Methods

Cytotoxicity Study

Normal human dermal fibroblasts were cultured in Eagle's minimal essential medium containing Earle's balanced salt solution supplemented with $1.5 \times$ essential amino acids and $1.5 \times$ vitamins (MEM Formula 785048 Gibco Laboratories, Grand Island, NY). The medium was completed with the addition of 10% fetal bovine serum (FBS, Gibco). Cells were grown in a Corning T-75 flask at 37°C under 95% air and 5% CO_2 in a Forma Scientific UN-I-TROL CO_2 Model 329 incubator (4, 5). Cultures were harvested by trypsinization (0.01% trypsin in 0.02% ethylenediaminetetraacetic acid (EDTA, Fisher Scientific, Fair Lawn, NJ) in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS) at confluency, seeded at a cell density of 1×10^5 cells per 100 mm Petri dish and incubated for 24 hr (expts. 1 and 2) and 48 hr (expt. 3). Cultures were then treated with 10 $\mu\text{g/mL}$ of either NIEHS chrysotile (mean length 21 μm ; mean width 0.17 μm ; or NIEHS crocidolite (mean length 10 μm ; mean width 0.27 μm) for 48 hr. At the end of the incubation, cultures were gently washed three times with 5 mL PBS and harvested by trypsinization. Three dishes per sample were used. Viable cell count was performed with the trypan blue exclusion method (5).

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Growth Kinetics of Chrysotile Exposed Human Fibroblasts

Fibroblasts were seeded at a density of 3.3×10^5 per 100 mm Petri dish. At the end of 9 hr incubation, growth medium was removed, and 10 mL growth medium was added to the cultures. The control group also received a change of the growth medium. Three dishes per group per time point were used to assess their viability at 3, 12, 28 and 36 hr post treatment.

^{14}C -Thymidine Incorporation

Cultures were seeded and treated in the same manner as described in expt. 3 of the cytotoxicity study. Prior to the cell harvesting, 2 $\mu\text{Ci}/\text{dish}$ of ^{14}C -thymidine (specific activity = 20 mCi/mmol, New England Nuclear, Boston, MA) was added to the cultures. The growth medium of each of the three dishes was removed. Cultures were gently washed three times with 0.5 mL PBS and trypsinized with 2 mL of trypsin. Addition of 3 mL of growth medium stopped the trypsin reaction. Cells were dispersed by mechanically pipetting five times with a 10 mL disposable plastic pipet. A 0.1 mL portion of the cell suspension was used for viable cell count. The remainder was pelleted by a brief centrifugation and washed with 5 mL $0.1 \times \text{SSC}$ at 4°C for 30 min. Macromolecules were precipitated with 10% cold trichloroacetic acid (TCA, Fisher). The precipitate was then washed once with cold methanol and dissolved in Soluene 100 (Packard Instruments, Downers Grove, IL) and counted in 10 mL of the scintillant, Aquasure (New England Nuclear, Boston, MA), with a Packard 3380 liquid scintillation spectrometer.

Results

The cytotoxic effects of asbestos treatment are summarized in Table 1. They were also evident by the 12 hr time point in Table 2, which shows growth kinetics of normal human fibroblasts during chrysotile exposure. At 3 hr after exposure to chrysotile, the viable cell count of the treated sample was significantly higher ($0.05 > p > 0.025$) than the control. This could be due to the fact that positively charged chrysotile was used by the fibroblasts as substrate for their anchorage at 3 hr. But by 12 hr, the cytotoxic effect of chrysotile was more important than any result of increased binding. Thymidine incorporation per viable cell rather than per culture dish was used to assess rate of DNA synthesis. By pulsing ^{14}C -thymidine at the last hour of the treatment, the effect of cell death was minimized. It is clearly

Table 1. Cytotoxicity of crocidolite and chrysotile asbestos on normal human fibroblasts.^a

Experiment	Viable cells/plate $\times 10^5$		
	Control	Crocidolite	Chrysotile
1	4.98 ± 0.22	2.74 ± 0.08	2.50 ± 0.15
2	4.48 ± 0.39	2.63 ± 0.27	2.98 ± 0.22
3	9.28 ± 0.58	6.50 ± 0.42	6.60 ± 0.42

^a 1.0×10^5 fibroblasts were seeded per 100 mm Petri dish at time zero. At 24 hr (expts. 1 and 2) and 48 hr (expt. 3), cultures were treated with 10 mL $10 \mu\text{g}$ asbestos/mL medium for 48 hr. Three dishes per sample were used. Values are viable cells/plate (mean \pm SD) $\times 10^5$.

Table 2. Effect of chrysotile treatment on growth kinetics of normal human fibroblasts.^a

Treatment time, hr	Viable cells/plate $\times 10^5$			<i>t</i> -test
	Control	Chrysotile		
3	2.34 ± 0.39	3.61 ± 0.64	0.05	$> p > 0.025$
12	2.72 ± 0.30	1.85 ± 0.21	0.025	$> p > 0.01$
28	3.79 ± 0.21	4.99 ± 2.83		NS
36	8.63 ± 0.96	7.13 ± 0.47		NS

^a 3.3×10^5 fibroblasts per 100 mm Petri dish were incubated for 9 hr prior to $100 \mu\text{g}/10 \text{ mL}$ chrysotile treatment for the time indicated. Three dishes per time point per sample were used. Values are viable cells/plate (mean \pm SD) $\times 10^5$.

Table 3. Effects of asbestos treatment on thymidine incorporation.^a

	cpm/cell $\times 10^5$	% of control
Control	1078.6 ± 51.7	100
Crocidolite	2104.4 ± 41.6	195
Chrysotile	1800.7 ± 73.8	167

^a 1.0×10^5 fibroblasts were incubated for 48 hr per 100 mm Petri dish prior to medium change to $100 \mu\text{g}$ chrysotile in 10 mL medium for another 48 hr. ^{14}C -Thymidine (2 $\mu\text{Ci}/\text{dish}$) was applied during the last hour of treatment. Three dishes per group were used. Thymidine incorporation was determined as radioactivity in TCA precipitate per viable cell (cpm/cell $\times 10^5$) \pm SD.

demonstrated in Table 3 that both crocidolite and chrysotile stimulated DNA synthesis in normal human dermal fibroblasts at the concentration studied. The enhancement by crocidolite was 25% more than chrysotile ($0.02 > p > 0.01$).

Discussion

It appears from these studies that asbestos exposure may facilitate the expression of a previously initiated cell by enhancing cellular proliferation. Crocidolite and chrysotile asbestos both induce cytotoxicity (Table 1) but not growth inhibition or re-

productive failure. The observed increased viable cells from 12 hr post treatment to 28 hr after exposure indicate a higher proliferation in treated compared to control cultures and the potentiated proliferation induced by treatment overcomes the cell killing effect of these mineral fibers at the concentration level examined. This analysis was further substantiated by the data showing enhanced thymidine incorporation shown in Table 3. Previously, we have also reported that an enhanced thymidine incorporation was observed with chrysotile exposure, when 2.5×10^5 fibroblasts per dish were plated for 24 hr before the treatment. This enhancement was also demonstrated in the presence of a nontoxic dose of benzo(a)pyrene (5). The lack of adsorption of thymidine on either asbestos type tested [for chrysotile (5) and for crocidolite (data are not shown)] suggested that the enhanced thymidine incorporation was not likely a result of an enhanced thymidine uptake due to adsorption and transport onto the cell by the fibers. The enhancement of proliferation by crocidolite is higher than that seen with chrysotile, which is consistent with the relative carcinogenicity of the two fibers in humans (7). These results are also consistent with those of Mossman et al. (2), who showed a higher DNA content in their crocidolite-treated epithelial cultures sooner than in their chrysotile-treated ones (3 days vs. 6 days). A factor in these results, however, may be some difference in the cytotoxicity of the two fibers. Also when Mossman et al. measured DNA content per culture dish under the influence of chrysotile, they observed a higher DNA content of the treated versus control only at day 6 after the treatment. Finally, a hyperplasia in hamster tracheal organ culture was also reported by Frank (8) at 6 days post treatment with amosite, another carcinogenic asbestos fiber. These results are compatible with our observations and interpretation that there are two competing processes going on within a biological system after its exposure to

asbestos: a cell-killing and a stimulation of DNA replication and suggest that asbestos exposure is participating in both cell killing and concurrently facilitating the expression of cellular transformation by stimulation of cellular proliferation. However, this study did not exclude the possibility that asbestos exposure is also an initiator of tumorigenesis.

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